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Deactivation of Macrophages with Interleukin-4 Is the Key to the Isolation of *Tropheryma whippelii*

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Whipple's disease is a systemic illness caused by a specific agent. Despite recognition of bacteria in lesions, efforts to isolate the causative agent remained futile. A novel strategy was devised to culture Whipple bacilli in deactivated mononuclear phagocytes. Infected tissue was inoculated into human phagocytes deactivated with interleukin (IL)-4, IL-10, and dexamethasone. Within 8–10 days, diastase-resistant periodic acid–Schiff–positive inclusions appeared, corresponding to intact and degenerating bacteria shown to be *Tropheryma whippelii* by electron microscopy and molecular analyses. *T. whippelii* was passaged several times in deactivated monocytes and a monoblastic cell line. Time-kinetics growth studies and comparative polymerase chain reaction analysis documented multiplication of *T. whippelii* in deactivated macrophages. Complementary studies showed that IL-4 rendered phagocytes permissive for *T. whippelii*, a strong indication that host factors contribute to the pathogenesis of Whipple's disease. The propagation of *T. whippelii* will permit microbiologic, immunologic, seroepidemiologic, and therapeutic studies of this pathogen.

Whipple's disease is a chronic infection involving the intestinal tract, lymph nodes, endocardium, central nervous system, skin, and other organs [1]. Although bacteria were recognized in lesions >30 years ago [2], recently the causative bacterium was identified by amplification of 16S rRNA genes in infected tissue, leading to the definition of a new bacterial species, *Tropheryma whippelii* [3, 4].

The lesions in Whipple's disease are characterized by the presence of large, foamy macrophages containing periodic acid–Schiff (PAS)–positive diastase-resistant inclusions, often with a typical rod shape [1, 5]. Untreated, the infection presumably results in the death of most affected individuals. Treatment of Whipple's disease remains based on empiric clinical grounds. Prolonged treatment with various antibiotics, while difficult, seems to be successful [1]. Immunologic defects have been postulated to predispose patients to develop Whipple's disease [6, 7], but it might well be that the occurrence of the infection depends entirely on the distribution of the pathogen in the human environment. While diagnosis of the disease is now greatly facilitated by the recently developed molecular techniques, the causative agent remains to be isolated and cultured.

Because we defined in our laboratory conditions permitting microorganisms to survive and grow in human phagocytes [8–

11], we decided to study whether *T. whippelii* could multiply within deactivated human macrophages. The deactivating agents dexamethasone, interleukin (IL)-4, and IL-10 all allow intracellular microorganisms such as *Listeria monocytogenes* to multiply in mononuclear phagocytes without affecting phagocytosis. Inoculation of tissue infected with *T. whippelii* into such deactivated human blood monocytes appeared, therefore, a promising strategy for the isolation and culture of this pathogen. Macrophages deactivated for 48 h with a maximally active combination of dexamethasone, IL-4, and IL-10 would ingest tissue debris with bacteria, but their killing systems would be crippled [10, 11], thereby permitting intracellular survival and eventually multiplication of the Whipple bacilli.

Methods

Patient material. For preliminary experiments, frozen (–70°C) intestinal biopsy material was obtained from a female patient with histologically proven Whipple's disease with spondylodiscitis, described previously [12]. Infected heart valves submitted for conventional bacterial cultures and eubacterial polymerase chain reaction (PCR) were from 2 male, white, native Swiss patients who underwent heart surgery for aortic insufficiency.

In patient TWZ1, infectious endocarditis was not suspected preoperatively. The diagnosis of infectious endocarditis was made by eubacterial PCR (see below). Subsequent to the heart operation, a duodenal biopsy showed, in addition to an inflammatory infiltrate, typical diastase/PAS–positive, silver stain–positive, acid fast–negative bacterial inclusions identical to those found in the heart valve. Blood cultures and cultures of the heart valve remained negative by conventional culture technique. Patient TWZ2 was diagnosed with culture-negative endocarditis. In retrospect, his history was compatible for seronegative arthritis of 21 years' duration and diarrhea for at least 4 years. A proximal duodenal biopsy

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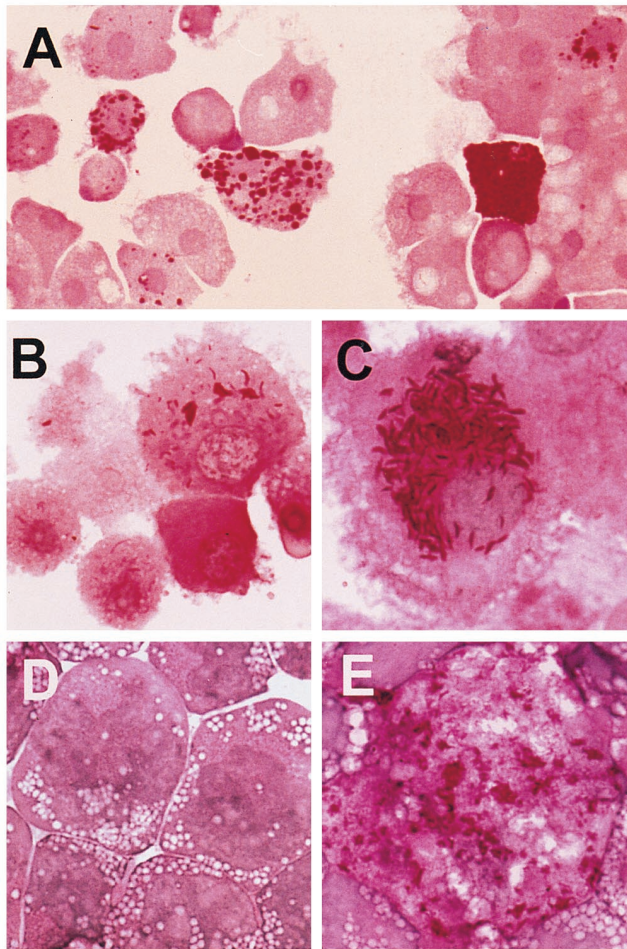


Figure 1. Growth of *T. whippelii*, isolated from heart valve biopsies of patients (TWZ1 or TWZ2), in cultured human macrophages and monoblasts. **A**, Periodic acid–Schiff (PAS)–positive diastase-resistant inclusions in deactivated human macrophages 10 days after inoculation with ground heart valve material (TWZ1, 1st passage). **B**, PAS-positive diastase-resistant inclusions and slender PAS-positive rods indicative for multiplication of *T. whippelii* in deactivated human macrophages 9 days after inoculation with infected macrophages (TWZ1, 2nd passage) shown in **A**. **C**, Formation of numerous PAS-positive rods in macrophages deactivated with IL-4 alone (TWZ2, 5th passage). **D** and **E**, Propagation of passaged *T. whippelii* in human monoblast cell line deactivated with IL-4. **D**, Uninfected SigM5 monoblasts. **E**, Infected, large, foamy monoblast showing numerous PAS-positive inclusions and rods (TWZ1, 3rd passage).

specimen obtained after 6 weeks of antibiotic therapy was negative for PAS-positive inclusions, but his heart valve was loaded with PAS-positive, acid fast–negative bacilli. In both patients, the diagnosis of *T. whippelii* infection of the heart valves was established by applying a eubacterial broad-range PCR, followed by sequencing of the amplicon as described [12] and by *T. whippelii*–specific PCR.

For patient TWZ1, a stretch of 384 bp was sequenced with 2 mismatches in comparison with the reference sequence of *T. whippelii* (3 instead of 2 Cs around position 60 and 1 instead of 2 Cs

around position 355 of the reference sequence). The next most closely related species was *Aureobacterium liquefaciens* (accession no. X77444), with a similarity of 89.1%. For patient TWZ2 the 398-bp sequence determined was identical to the reference sequence of *T. whippelii* (accession no. M87484) except for 2 ambiguous bases (not mismatches) in patient TWZ2's sequence. The next most closely related sequence was again *A. liquefaciens*, with a similarity of 89.5%. In both cases, the diagnosis was further corroborated by species-specific PCR using both the primer pairs TW-1/TW-2 and TW-1/TW-3 on DNA extracted from the valves. Intestinal biopsies from patient TWZ1 also gave a positive species-specific PCR result [4].

Human mononuclear phagocytes. Human mononuclear phagocytes were purified from peripheral blood as described, giving >90% monocytes with <10% contaminating lymphocytes and only insignificant numbers of neutrophils [10, 11]. Cytokines or dexamethasone was added after cells were incubated at 37°C for adhesion for 2 h and washed three times for the removal of nonadhering cells. The monoblast cell line SigM5 was established from exponentially growing blasts of bone marrow from a patient with M5A-type myeloid leukemia. The cell line stably expresses the monocytic markers CD13, CD14, CD15, CD33, and CD45 (Pan leukocyte) by flow cytometry and has no B and T lymphocyte markers. By karyotype analysis, the cell line shows a stable trisomy 8.

Inoculation of cell cultures. Before infection, primary macrophages and monoblast cells were incubated with deactivating cytokines or dexamethasone as indicated for 48 h in 6-well tissue culture plates or 500-mL culture flasks, respectively (large-volume cultures; Falcon, Oxnard, CA) at 0.5×10^6 cells/mL in Iscove's modified Dulbecco's medium (GIBCO Europe, Basel, Switzerland) supplemented with 20% human pooled serum and 2 ng/mL human recombinant (hr) IL-4 (specific activity $>2 \times 10^6$ U/mg; Collaborative Biomedical, Becton Dickinson, Bedford, MA), 5 ng/mL hrIL-10 ($ED_{50} = 2$ ng/mL in MC-9 costimulation assay; Pepro Tech, Rocky Hill, NJ), and 2.5×10^{-7} M dexamethasone (Sigma Chemie, Buchs, Switzerland) in an incubator (SteriCult; Forma Scientific, Marietta, OH) at 37°C, 5% CO₂, and 98% humidity. No antibiotics were added.

For primary infection of cell cultures, homogenized material from ~1 mg of heart valve tissue was suspended in 500 μ L of 0.9% NaCl. Aliquots (2 μ L) of this suspension were added to the wells of the 6-well plates with deactivated primary phagocytes or monoblast cells in a volume of 2 mL of complete medium. Infected cultures were further incubated for 8–10 days under the conditions described above without replacement of the medium. Then cells were collected together with the culture medium (2 mL), and aliquots of these cell suspensions were analyzed for *T. whippelii* by diastase/PAS staining of cytocentrifuge preparations (100- μ L aliquots), electron microscopy (1.5-mL aliquots), and PCR (300- μ L aliquots) or were used as secondary inocula (200 μ L/2 mL/well) for further passages of *T. whippelii* in cultured cells as described above.

For expansion of *T. whippelii* in a large volume of cells, 1 mL of infected cell suspension after the second passage was inoculated into 50 mL of deactivated SigM5 cells at a density of 2×10^6 cells/mL on a 250-cm² growth area. This culture was further incubated for 9 days; the cell suspension was then analyzed by PAS

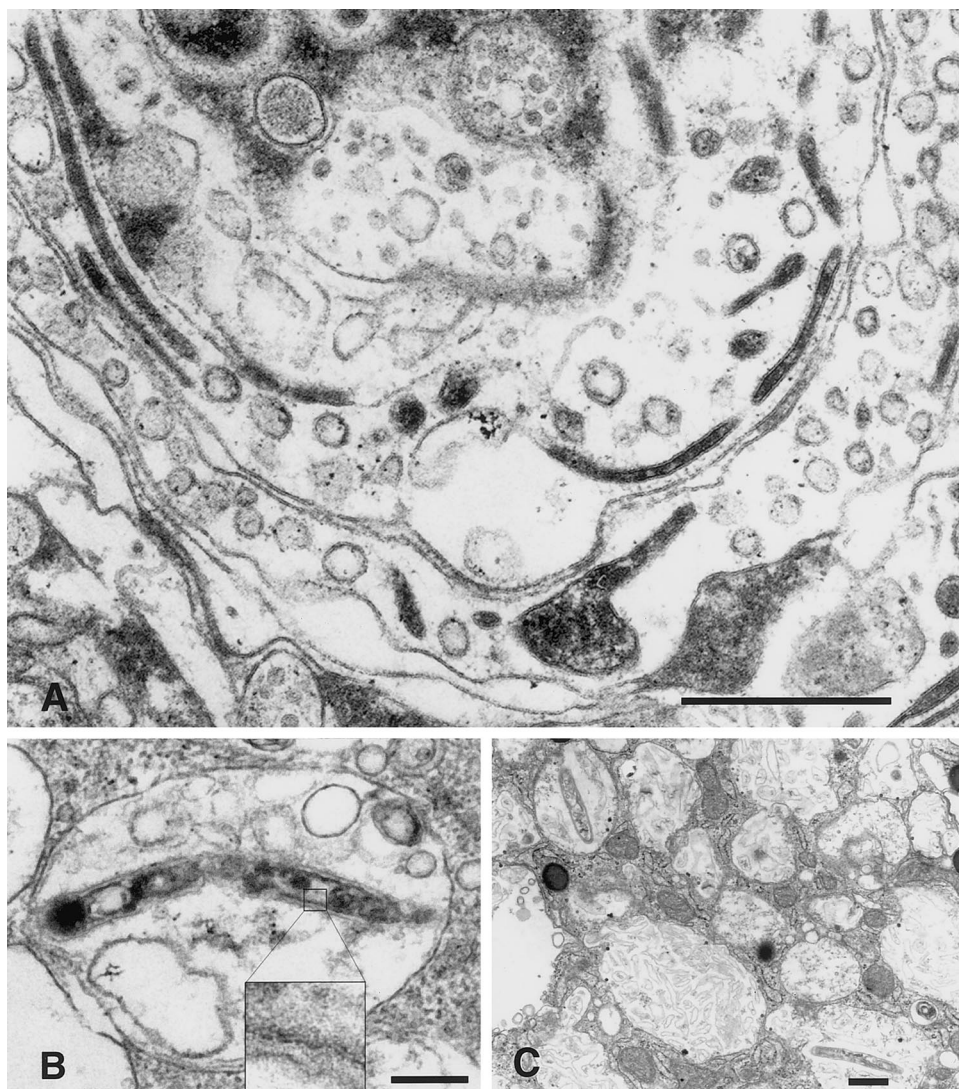


Figure 2. Electron micrographs showing Whipple bacilli cultured in deactivated human macrophages and SigM5 monoblast cells. **A**, Macrophage deactivated with IL-4 and infected with passaged inoculum (TWZ2, 3rd passage) showing numerous slender bacteria in longitudinal and transverse sections. Bar = 450 nm; original magnification, $\times 12,000$. **B**, SigM5 monoblast deactivated with IL-4, IL-10, and dexamethasone and infected with TWZ2 inoculum (2nd passage) showing bacillus within vesicular cellular structure. Insert: Magnification of part of cell wall with trilaminar structure unique to cell wall of *T. whippelii*. Bar = 300 nm; original magnification, $\times 9050$. **C**, Deactivated human macrophage infected with TWZ1 inoculum (5th passage). Bacilli range from normal appearance to advanced stages of digestion; latter consists of cell wall remnants clustered in large vesicles, referred to as Whipple bodies, in light microscopy of periodic acid–Schiff–stained cells. Bar = 450 nm; original magnification, $\times 4200$.

staining, electron microscopy, and PCR as described above or frozen at -70°C in 1-mL aliquots and used for further experiments.

Electron microscopy. For electron microscopy, cell suspensions collected 9 days after challenge were fixed in 3% glutaraldehyde and processed using standard methods [10].

Molecular analyses. For PCR analysis, 300- μL aliquots of cell suspensions collected 9 days after infection were centrifuged, and the DNA was extracted by digesting the samples for 3 h at 55°C in 70 μL of 50 mM TRIS-HCl (pH 8.5), 1 mM EDTA, 0.5% SDS, and 200 $\mu\text{g}/\text{mL}$ proteinase K. After proteinase K was inactivated at 95°C for 12 min, 2 μL was directly amplified in a PCR mix containing 2% Tween 20 and primer pair TW-1/TW-3 or TW-1/TW-2 as described [12], resulting in fragments of 141 and 267 bp, respectively. The number of cycles was always 40. After Southern blotting to a nylon membrane, hybridization was done using the internal digoxigenin-labeled oligonucleotide TW-4 (5'-CACGGT-TGTCGTCAGCTCGT-3') at 50°C overnight. The positive control was intestinal biopsy material from a patient with confirmed Whipple's disease known to contain *T. whippelii* DNA, as shown by

PCR using primers pW3FE and pW2RB (corresponding to our TW-1 and TW-3 primers) [4, 12, 13].

Statistical analysis. For statistical analysis, the *t* test with Dunnett's posttest analysis for the comparison of several groups with one control was performed with a commercial software package (Instat; Graphpad, San Diego).

Results

In a first series of isolation attempts with intestinal biopsy material from a patient with Whipple's disease, we failed because of inevitable bacterial overgrowth. We then were able to obtain infected heart valve material from 2 patients undergoing heart surgery for endocarditis. Crushed tissue samples from each patient (TWZ1 or TWZ2) were inoculated into cultures of human monocytes deactivated with a combination of dexamethasone, IL-4, and IL-10. These infected cells were subse-

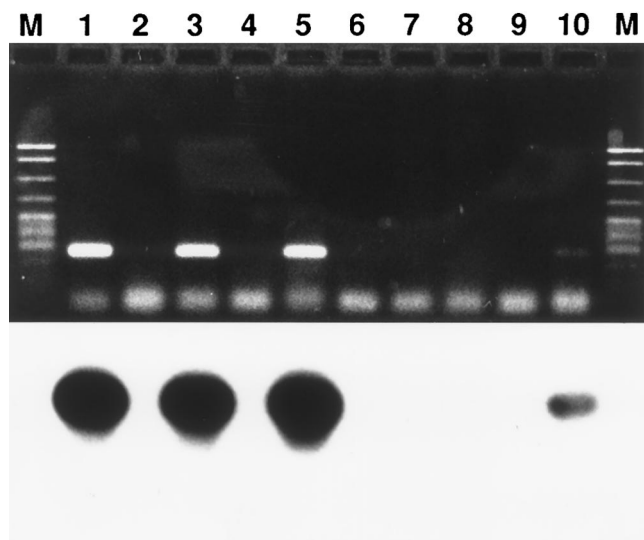


Figure 3. Specific detection of *T. whippelii* DNA in cell cultures by polymerase chain reaction (PCR). Top, agarose gel electrophoresis and ethidium bromide staining; bottom, Southern blot hybridized with digoxigenin-labeled internal oligonucleotide TW-4. Lanes M: molecular weight marker (pBR322 DNA digested with *MspI*), fragment size (from top), 622, 527, 404, 307, and 240 bp down to <50 bp. Lane 1: human blood-derived macrophages inoculated with heart valve material of patient TWZ1. Lane 2: uninfected macrophages. Lane 3: 2nd passage of isolate TWZ1 in SigM5 cells (monoblast cell line described in figure 1). Lane 4: uninfected SigM5 cells. Lane 5: 3rd passage of isolate TWZ1 in large-volume cultures of SigM5 cells. Lane 6: uninfected SigM5 cells from large-volume cultures. Lanes 7 and 8: small bowel biopsies from patients without Whipple's disease. Lane 9: PCR reagents only (no DNA added). Lane 10: Clinical specimen (intestinal biopsy) known to contain *T. whippelii* DNA (supplied by M. Maiwald, Germany) [13].

quently used to further passage *T. whippelii* in deactivated human macrophages and monoblast cells. Within 8–10 days after infection, macrophages appeared filled with coarse, diastase-resistant, PAS-positive conglomerates and slender PAS-positive rods (figure 1) that were both acid fast–negative (not shown). Heating of inocula at 65°C for 2 h precluded the appearance of PAS-positive inclusions (not shown).

By electron microscopy, it was confirmed that these inclusions corresponded to intact and degenerating bacteria (figure 2) described in biopsy material from patients with Whipple's disease [1, 14]. In contrast to findings with tissue specimens, however, extracellular bacteria were never seen by any method we tested, indicating the cellular environment is required for bacterial growth, and PCR analyses of culture supernatants remained negative. PCR studies with specific primers confirmed that the isolated bacteria from both patients were indeed *T. whippelii* (figure 3 for patient TWZ1). To establish stable propagation of *T. whippelii*, we expanded the bacterial population of both isolates after two passages in large-volume cultures of deactivated SigM5 monoblasts (figure 1D–E; figure 3, lane 5). After freezing and storage at –80°C, such passed bacteria

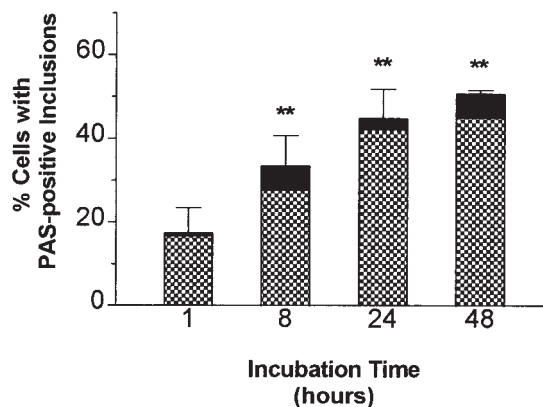


Figure 4. Time-kinetics of growth of *T. whippelii* in deactivated human macrophages. Macrophages plated in 24-well tissue culture plates were deactivated with IL-4 and IL-10 for 48 h prior to infection with 100 μ L of freshly defrosted aliquot of large-volume culture of TWZ1 (3rd passage) in SigM5 cells. At indicated times, cells were collected, cytocentrifuge preparations were stained with diastase/periodic acid–Schiff, and infected cells were counted. Total bar = before diastase treatment, hatched area = after diastase digestion (diastase-resistant). Data are mean \pm SD of duplicate preparations from triplicate wells. ** $P < .001$.

remained infective for deactivated monocytes and were used for further experiments.

Kinetics growth studies. Next we turned to kinetics growth studies for demonstration of multiplication of a defined inocu-

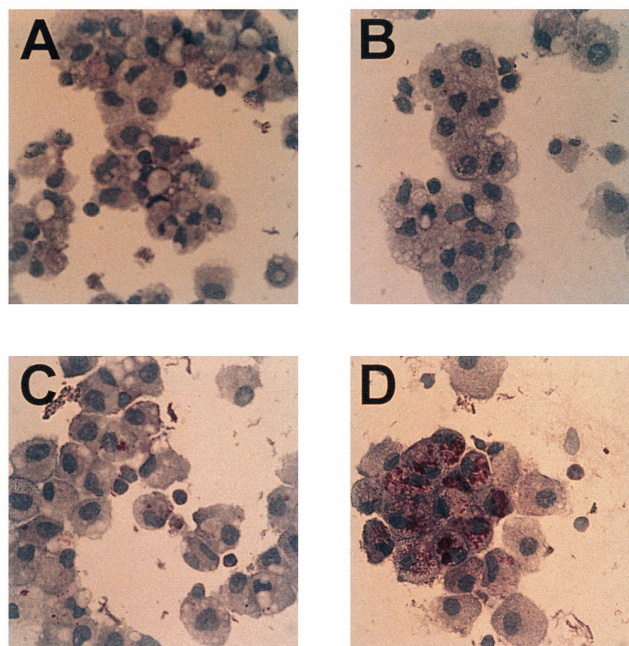
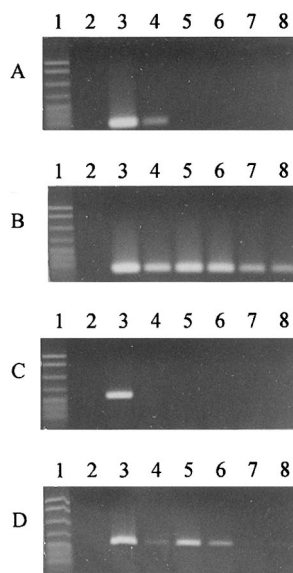


Figure 5. Microphotographs of cytospin preparations corresponding to figure 4 showing time-dependent increase of periodic acid–Schiff–positive inclusions. A, 1 h. B, 8 h. C, 24 h. D, 48 h.

Figure 6. Comparative polymerase chain reaction analysis of consecutive passages of original heart valve inoculum TWZ1 (**B, D**) and corresponding dilutions of uncultured inoculum (**A, C**). **A** and **B** compare inoculum (**A**) with culture passages (**B**) using primer pair TW-1/TW-3, yielding 141 bp. **C** and **D** compare inoculum (**C**) with culture passages (**D**) using primer pair TW-1/TW-2, yielding 267 bp. Lanes 1: molecular size markers pBR322 DNA digested with *MspI* (for sizes, see figure 3). Lanes 2, negative control (DNA from uninfected human macrophages). Lanes 3–8: dilutions of original inoculum corresponding to dilutions during consecutive passages (by lane): 1:10,000 (3), 1:500,000 (4), 1:5 × 10⁶ (5), 1:2 × 10⁷ (6), 1:8 × 10⁷ (7), 1:3.2 × 10⁸ (8). Comparison of corresponding dilutions infers multiplication by factor of at least 100–1000 during 4 passages of original inoculum in human phagocytes.



lum in phagocytes (figures 4, 5). These experiments showed a time-dependent augmentation of acid fast-negative, diastase-resistant, PAS-positive inclusions in deactivated macrophages (figures 4, 5). To further confirm the multiplication of *T. whippelii* in consecutive passages of the original inoculum, passaged isolates of 1 patient (TWZ1) were compared with the corresponding dilutions of the original heart valve material without incubation. As shown by PCR analysis (figure 6), the different yields of copies in the cultured versus uncultured inoculum were already clearly visible at passage 3, corresponding to a dilution of 1:500,000 (figure 6, lanes 4). Higher dilutions of the original inoculum no longer yielded amplified products (figure 6A, C), while the corresponding passages were still positive (figure 6B, D).

Requirement of macrophage deactivation for intracellular growth. We then studied the individual effects of the three deactivating signals initially used in combination for the isolation of *T. whippelii* (figure 7). We found that IL-4, previously shown to be the most effective deactivating agent in the *Listeria* model [10], was the critical deactivating signal that promoted intracellular multiplication of *T. whippelii*. In contrast to results from studies with *L. monocytogenes* [10], IL-10 and dexamethasone did not significantly promote intracellular multiplication. To the contrary, dexamethasone interfered with the propagation of *T. whippelii* in macrophages (figure 7). Identical observations were made with the monocytic human cell line (not shown).

Discussion

We report here the first isolation and propagation of *T. whippelii* in human monocytes and monoblasts after deactivation

with IL-4. The method chosen for the support of growth of this bacillus was proven to be reproducible with infected material from 2 patients. Growth-kinetics studies of passaged isolates revealed a time-dependent increase of diastase/PAS-positive inclusions within macrophages. Multiplication of *T. whippelii* in our culture system was further corroborated by comparative PCR analysis of passaged isolates with corresponding dilutions of the original (uncultured) inoculum.

The novel, yet simple strategy used for isolation of this uncultured microorganism should also be tried for the isolation of other uncultured or unidentified microorganisms, such as *M. leprae*, or even suspected agents in diseases, such as Crohn's disease, sarcoidosis, Wegener's granulomatosis, or SAPHO (synovitis, acne, palmoplantar pustulosis, hyperostosis, osteitis) syndrome.

In contrast to observations in histologic studies of tissues infected with *T. whippelii* [1], intracellular bacteria were observed only in cytospin preparations in our study, indicating that *T. whippelii* is an intracellular pathogen, and PCR analysis of supernatants with both primer sets produced no amplicons. The requirement of IL-4, an immunoregulatory cytokine produced by various immune cells, notably Th2 cells [15], indicates a probable contribution by host factors to the pathogenesis of Whipple's disease, such as an imbalance in Th1-Th2 immune response. While the target for IL-4 in the mononuclear phagocyte has not yet been elucidated [10, 11], IL-4 clearly renders monocytes permissive for intracellular growth of mi-

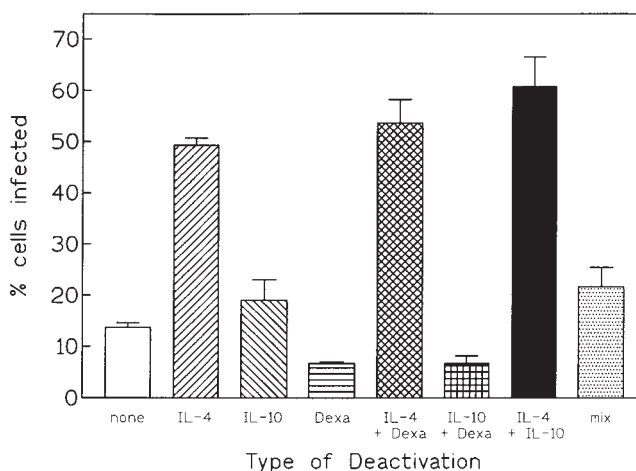


Figure 7. Effects of single and combined deactivating agents (IL-4, IL-10, dexamethasone) on formation of periodic acid-Schiff (PAS)-positive inclusions corresponding to growth of *T. whippelii* in human macrophages infected with TWZ2 inoculum, which was passed twice through macrophages deactivated with combination of all 3 signals. Results are % of macrophages showing typical PAS-positive inclusions after incubation for 9 days in continuous presence of deactivating agents. Only IL-4 (2 ng/mL) had significant deactivating effect ($P < .001$); IL-10 (5 ng/mL) had no effect ($P > .1$). Dexamethasone (2.5×10^{-7} M), with exception of combination with IL-4, suppressed propagation of *T. whippelii*. Mean \pm SE from 3 independent experiments with phagocytes from 3 donors.

croorganisms in vitro [10, 11] and in vivo leads to an increased susceptibility of cells to intracellular pathogens such as *Listeria* [15] and *Leishmania* organisms [16].

In any event, the isolation and culture of *T. whippelii* described herein will permit further studies on the pathogenesis, immunology, and therapy of this intriguing infection.

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